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L-deprenyl, a monoamine oxidase-B inhibitor, has been reported to reverse the age-related decline in sympathetic noradrenergic innervation and immune function in old rats and enhance cell-mediated immunity in tumor-bearing rats. The aim of the present study was to investigate whether deprenyl treatment of old female rats with spontaneously developing mammary tumors could increase sympathetic noradrenergic activity and immune responses to inhibit the tumor growth. Female Sprague-Dawley rats with spontaneous mammary tumors were administered 0, 2.5 mg, or 5.0 mg/kg body weight (BW)/day deprenyl for i.p. 9 weeks. Tumor diameter, tumor number and body weight were measured throughout the treatment period. At the end of the treatment period, norepinephrine (NE) concentration, interferon-γ production (IFN-γ), Con A-induced T lymphocyte proliferation, and percentage of T and B lymphocytes and natural killer cells were measured in the spleen, and the concentrations of monoamines were measured in the medial basal hypothalamus. Treatment with deprenyl reduced tumor growth, increased NE concentration, IFN-γ production and percentage of the CD8+ T lymphocytes in the spleen in comparison to saline-treated rats. In the medial basal hypothalamus, deprenyl treatment increased the concentrations of catecholamines and indoleamine. These results suggest that the anti-tumor effects of deprenyl on spontaneous rat mammary tumors may be mediated through neural-immune signaling in the spleen and medial basal hypothalamus.

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David S. Tulton, MD, PhD

PI - Signature Date

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Introduction

The development and growth of spontaneous mammary tumors in aging female rats is associated with an increase in prolactin (PRL) and a decrease in ovarian hormone secretion (1). PRL secretion from the anterior pituitary is controlled by tuberoinfundibular dopaminergic (TIDA) activity in the medial basal hypothalamus (MBH). Agonists and antagonists to DA release promoted or suppressed the development and growth of tumors, respectively, indicating that mammary tumorigenesis is contingent upon the availability of PRL (1, 2). Besides these neuroendocrine changes, inhibition of immune functions facilitates development and growth of tumors. Tumorigenesis is also influenced by cytokines that regulate macrophage, T- and B-lymphocyte activation, and activities of natural killer (NK) cells and lymphokine-activated killer cells (3). The sympathetic nervous system through norepinephrine (NE) can modulate cytokine production and other activities of the immune system (4). Thus, the sympathetic noradrenergic (NA) system may play a role in modulating tumor-specific immune responses.

Previously, we have shown that L-deprenyl, a monoamine oxidase-B (MAO-B) inhibitor, suppressed development and growth of tumors by lowering PRL secretion through enhanced hypothalamic TIDA activity in rats with carcinogen-induced mammary tumors (5, 6). Long-term administration of deprenyl to old female rats inhibited the incidence of spontaneously developing mammary tumors by increasing DA in the MBH and decreasing serum PRL (7). Deprenyl has been used in the treatment of human neurodegenerative disorders, Parkinson's disease and Alzheimer's disease, due to its ability to improve central neuronal functions (8-10). Our laboratories have reported that treatment of old rats with deprenyl reversed the age-related decline in NA innervation of the spleen and also increased NK cell activity and IL-2 production, indicating that deprenyl is capable of altering functional activities of both the nervous and immune systems (11-12). A recent study revealed similar increase in NK cell activity, IL-2 and IFN-γ production, sympathetic NA activity in the spleen, and TIDA activity in the MBH of deprenyl-treated rats with carcinogen-induced mammary tumors (unpublished data). The present study was conducted to investigate whether deprenyl can inhibit tumor growth in intact old female rats with spontaneously developing mammary tumors.

Materials and Methods

Animals. Female Sprague-Dawley rats (12 mo-old) were purchased from Charles River Laboratories, Kingston, NY and housed individually in a temperature-controlled and light-controlled (12:12 h light/dark cycle) animal room. All animals received food and water ad libitum. The animals were palpated for the presence of tumors at the time of arrival and thereafter every week for the development of tumors. No tumors were detected at the time of arrival. After 7-8 months, animals started developing mammary tumors.

Treatment. After the appearance of tumors (1-2 cms in diameter), the rats were randomly divided into three different groups that received either saline (n=10), 2.5 mg (n=12) or 5.0 mg (n=11) of deprenyl/kg BW/day i.p. for 9 weeks. R(-)-Deprenyl hydrochloride was purchased from RBI, Natick, MA. Tumor diameter, tumor number, and body weight were measured every week throughout the treatment period. Tumor diameter was calculated by averaging two perpendicular diameters measured by vernier calipers. Percent change in tumor diameter was calculated using the equation, (Average diameter in cms $_{\text{week n}}$- Average diameter in

cms $_{week\ 0}$) X 100. At the end of the treatment period, the animals were sacrificed, and the MBH were rapidly removed and frozen immediately on dry ice. Spleens were removed aseptically and cut into three blocks; two out of three blocks were frozen on dry ice, and stored at -80°C until further analysis for high performance liquid chromatography with electrochemical detection (HPLC-EC). The third block of spleen was used for immunological assays including IFN-γ production, Con A-induced proliferation of T lymphocytes, and flow cytometry.

Lymphocyte preparation. Lymphocytes from the spleen were prepared as described previously (12, 13). Cells were resuspended to the desired concentration in RPMI 1640 medium supplemented with 5% fetal calf serum (Sigma), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.01 mM nonessential amino acids, 5 x10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, 24 mM sodium bicarbonate, and 10 mM HEPES for in vitro culture.

Assay for IFN-γ production. Lymphocytes (2 X 10^5 cells/well) were incubated with either medium alone or 1.25 μg/ml of Con A in 24-well tissue culture plates (Falcon, Becton Dickinson, Oxnard, CA). After 24 h of culture, 1 ml of supernatant was removed from each well and stored at -20°C until assayed for cytokine content.

IFN-γ levels in supernatants were determined by ELISA. ELISA plates (Corning, Corning, NY) were coated overnight at 4°C with purified anti-rat IFN- γ polyclonal Ab (1 μg/ml; Biosource International, Camarillo, CA) in 0.1 M Na_2HPO_4 buffer (pH 9.0). In between steps, plates were washed with PBS containing 0.05% Tween-20 (PBS/Tween). Plates were then blocked for 2 h with PBS-10% fetal equine serum (FES) at room temperature. Recombinant rat IFN- γ (Biosource) or samples serially diluted in culture media were added to plates in triplicate and incubated overnight at 4°C. Biotin-conjugated anti-rat IFN- γ (0.5 μg/ml; Biosource), diluted in PBS-10% FES, was added and the plates were incubated at room temperature for 1 h. Avidin-peroxidase (Sigma), diluted 1:400 in PBS-10% FES, was added to the plates, and incubated for 30 min at room temperature. In the final step, substrate ABTS (2,2'-Azino-bis(3-ehylbensthiazoline-6-sulfonic acid; Sigma) containing 0.03% hydrogen peroxide was added to the plates and incubated for 30 min at room temperature. Absorbance at 405 nm was measured with a microplate reader (Bio-Tek instruments) after 30 min. The amount of IFN- γ in samples was determined by extrapolation to the standard curve.

Con A-induced proliferation. Spleen cells, 2 X 10^5 cells/well, were cultured in triplicate with either medium alone or varying concentrations of Con A (Calibiochem-Behring Corp., La Jolla, CA), in 96-well, flat bottom tissue culture plates (Falcon), and maintained for 3 days at 37°C in 5% CO_2-humidified incubator. [^{3}H]-Thymidine (0.5μCi/10μl; 5 Ci/mmol; DuPont NEN, Boston, MA) was added for the final 18 h of culture. Cells were harvested on to glass fiber filter paper (Whatman Inc., Clifton, NJ) with a cell harvester (Skatron). The dried filters were placed in scintillation fluid (Biosafe II, RPI, Mount Prospect, IL), and radioactivity determined with a liquid scintillation counter (LKB, Wallac, Finland).

Flow cytometric analysis. Spleen cells were washed in PBS containing 2% BSA and 0.02% azide (flow wash). Fluorescein-conjugated anti-rat sIgM (clone G53-238, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-NKR-P1A (an NK cell marker, clone 10/78, diluted 1:40; Pharmingen) or fluorescein-conjugated anti-rat CD8 (clone OX-8, diluted 1:40; Pharmingen) and phycoerythrin-conjugated anti-CD4 (clone OX-35, diluted 1:20; Pharmingen)

were added to 2 X 10^6 cells and incubated at 4°C for 30 min. Cells incubated with flow wash alone were included to determine autofluorescence. Following this incubation, cells were washed twice in flow wash, fixed in PBS containing 1% paraformaldehyde, and stored in the dark for no longer than 2 weeks at 4°C prior to analysis. Two-color fluorescence was analyzed with an Elite flow cytometer (Coulter Electronics, Hialeah, FL), equipped with an argon-laser at 15 mW and excitation wavelength of 488 nM.

HPLC-EC. The HPLC-EC procedure has been described in detail before (6, 11, 12). Briefly, NE in the spleen was extracted with alumina prior to analysis by HPLC-EC. Tissues were homogenized in 0.1 M of $HClO_4$ with 0.25 μm of 3,4-dihydroxybenzylamine (DHBA) as the internal standard and were centrifuged at 1000*g* for 5 minutes. The supernatants were used for the aluminum oxide extraction while the pellets were saved for protein assay (Bio-Rad assay kit). For the estimation of monoamines in the MBH, a volume of 200 μl of $HClO_4$ containing 0.25 μm of 3,4-dihydroxybenzylamine (DHBA) was added as the internal standard to the tubes containing MBH. The tissues were sonicated and centrifuged for 2 min at 1000*g*. The supernatants were stored at -80°C until analyzed for the concentrations of NE, dopamine (DA), serotonin (5-HT), and their metabolites by HPLC-EC and the pellets were stored for the measurement of protein concentrations. At the time of HPLC-EC analysis, samples were loaded onto a Waters 717plus autosampler (Waters, Milford, MA). Splenic NE concentration was expressed in terms of both pmoles/mg protein and pmoles/mg wet weight of the tissue. NE content in the whole spleen was calculated using NE concentration/mg wet weight in the combined hilar and end region of the spleen (12). The neurotransmitter concentrations in the MBH were expressed in terms of pmoles/mg protein.

Statistical analysis. The data were analyzed by ANOVA. Con A-induced proliferation was analyzed using ANOVA with Con A concentration as repeated measures. Parameters that attained significance following ANOVA ($P<0.05$) were further analyzed by Fisher's least significant difference test.

Results

Mammary tumor size was similar with no significant differences among the three groups at the beginning of the treatment. As shown in Figure 1, tumor diameter increased significantly in the saline-treated group during the 9-week treatment period to more than 100% of the initial size at the end of the treatment period. In contrast to saline-treated rats, there was a significant inhibition of tumor growth in deprenyl-treated rats that was apparent from the fifth week of treatment. Among the 10 rats in the saline group, 7 rats had a consistent increase in tumor diameter and 3 rats showed a slight or no increase in tumor diameter. In contrast, in the 2.5 mg/kg deprenyl-treated group, the tumor diameter decreased in 5 rats, remained unaltered in 4 rats, and increased in 3 rats. Similarly, 6 rats showed a decrease in tumor diameter, 2 rats had no alterations in tumor diameter, and 3 rats had an increase in tumor growth in the 5.0 mg/kg deprenyl-treated group.

There were no significant differences in the number of tumors (Figure 2) and body weight (Figure 3) between the three groups during the 9-week treatment period.

IFN-γ was measured in supernatants obtained from Con A-stimulated splenocytes (Figure 4). IFN-γ production was significantly ($P<0.05$) increased in spleen cells from rats treated with 2.5 mg/kg deprenyl. In vitro Con A-induced T cell proliferation was unaltered among the three

treatment groups at suboptimal and optimal doses of mitogen, but it was significantly ($P<0.05$) higher in 5.0 mg/kg deprenyl-treated rats at 5.0 μg/ml of Con A (Figure 5). Deprenyl treatment induced no significant modification in the percentage of sIgM+ B cells, CD4+ T cells, and NK+ cells in the spleen (Table 1). However, there was a slight increase in the percentage of splenic CD8+ T cells of rats treated with 2.5 mg or 5.0 mg/kg deprenyl.

The concentration of NE (per mg protein and per mg wet weight) was elevated significantly ($P<0.05$) in the hilar regions of spleens from rats treated with 2.5 mg/kg and 5.0 mg/kg deprenyl in comparison to rats treated with saline (Table 2). Spleen weight was unaltered by deprenyl treatment.

The concentrations of NE and serotonin (5-HT) were significantly ($P<0.001$) higher in the MBH of rats that received 2.5 mg/kg and 5.0 mg/kg deprenyl (Table 3). The concentration of 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of 5-HT, was unaltered in the MBH of deprenyl-treated rats. The concentration of the dopamine (DA) metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), was significantly ($P<0.001$) lower in the MBH of deprenyl-treated rats. Treatment with 5.0 mg/kg increased ($P<0.001$) DA concentration in the MBH.

Discussion

The results from the present study demonstrate that deprenyl treatment prevents tumor growth and simultaneously enhances catecholaminergic activity in the MBH and spleen, and immune reactivity in the spleen of old female rats with spontaneously occurring mammary tumors.

The three major hormones that determine the development and growth of mammary tumors are prolactin (PRL), estrogen and progesterone, but other hormones including thyroid hormones, growth hormone, insulin and growth factors also can influence mammary tumorigenesis (1, 14). In rodents, an increase in PRL level significantly increases the incidence of spontaneously developing mammary tumors while a decrease in PRL level inhibits the development of these tumors (15). Treatment of old female rats with deprenyl for a period of 8 months reduced the incidence of spontaneously occurring mammary tumors and pituitary tumors in association with a decrease in PRL secretion and monoamine metabolism in the MBH (7). Acute administration of deprenyl to young female rats also reduced serum PRL, confirming the inhibitory effects of deprenyl on PRL secretion (16). In the present study, the concentration of DA, the principal inhibitory neurotransmitter of PRL secretion, is elevated following deprenyl treatment. An increase in dopaminergic activity in the MBH suggests that deprenyl may suppress PRL secretion through the release of DA into the anterior pituitary (5, 7, 17, 18). Serotonin in conjunction with tumor necrosis factor has been reported to prevent tumor growth by decreasing in blood flow to the tumors and inducing hemorrhagic necrosis (19, 20). It is possible that an increase in the concentration of serotonin in the MBH may exert similar anti-tumor effects on the growth of mammary tumors.

Preovulatory surge in estrogen and PRL secretion during each estrous cycle in young rats is neurotoxic to TIDA neurons resulting in the development of pituitary prolactinomas and age-associated development of mammary tumors. Ovariectomy and administration of ergot derivatives are known to reduce the incidence of tumors indicating that neuroprotection of TIDA neuronal system may aid, in part, preventing the development of tumors (21). Pre- and post-treatment of carcinogen treated rats with deprenyl prevented the development of mammary tumors, possibly through neuroprotection and an enhancement of the tuberoinfundibular

dopaminergic (TIDA) neuronal activity in the MBH (6). Several studies support the view that deprenyl is a neuroprotective and neurorestorative agent; deprenyl prevented diminution of tyrosine hydroxylase-positive nerve fibers in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice, and facilitated the regrowth of splenic sympathetic noradrenergic nerve fibers in young sympathectomized and old rats (11, 12, 22).

Lymphocyte proliferation, delayed-type hypersensitivity, and cytolytic and cytotoxic functions are also suppressed in tumor-bearing rats (3). Treatment of tumor-bearing rats with 2.5 mg/kg deprenyl increased splenic IFN-γ production while both doses of deprenyl increased the percentage of CD8+ T cells. The lack of alteration in splenic IFN-γ production in rats that were treated with 5.0 mg/kg deprenyl is not known suggesting that deprenyl's effect on cytokine levels may be dose-dependent, age, and strain of rats. Administration of deprenyl to rats with carcinogen-induced mammary tumors and to old male rats stimulated splenic IL-2 and IFN-γ production and increased NK cell activity (12, unpublished data). An increase in splenic IFN-γ production may be responsible for the activation of NK cells that are involved in restriction of tumor growth. The moderate increase in CD8+ T cells suggests that an anti-tumor effect of deprenyl may have been achieved, in part, through these effector cells critical to the destruction of tumor cells, but it is yet to be determined whether deprenyl can also enhance anti-tumor cytolytic activity.

An increase in immune responses following deprenyl treatment of tumor-bearing rats may be due to an increase in NE concentration in the spleen. Several lines of evidence indicate that NE modulates immune responses in spleen and lymph nodes. In young mice, destruction of NA nerve terminals by chemical ablation with 6-hydroxydopamine results in depletion of NE in the periphery and diminished T cell-mediated immune responses, including delayed hypersensitivity, cytotoxic T lymphocyte activity, Con A-induced T cell proliferation, and IL-2 and IFN-γ production (13, 23). Treatment of old male rats with deprenyl reversed the age-related decline of NA innervation in the spleen and also improved splenic NK cell activity and IL-2 production (12). Rats with carcinogen-induced mammary tumors had lower levels of splenic NE concentration, and deprenyl treatment restored NE content in the spleen (unpublished data). Collectively, the evidence indicate that immunosuppression may correlate with a decline in splenic NE content.

Key Research Accomplishments

- Deprenyl, a monoamine oxidase-B inhibitor, is an effective drug in arresting tumor growth in old rats that spontaneously develop mammary tumors
- The inhibition of tumor growth is achieved by enhancement of dopaminergic activity in the hypothalamus that also regulates prolactin secretion from anterior pituitary.
- Immune functions especially, IFN-g production and the percentage of CD8+ T cells in spleen were higher in deprenyl-treated rats.
- Splenic NE concentration was also higher indicating that neural-immune interactions are also important in determining the degree of tumorigenesis.

Conclusions

In summary, we have shown that deprenyl can inhibit mammary tumor growth. This reduced tumor growth is accompanied by an increase in hypothalamic DA, NE, and serotonin content, splenic NE concentration, and immune responses in old female rats with spontaneously developing mammary tumors. Deprenyl may inhibit tumor growth by several mechanisms.

These results suggest that one of the mechanisms may be through communication between the nervous system and the immune system to enhance anti-tumor immunity in tumor-bearing rats.

Future studies are necessary to determine whether deprenyl is capable of influencing cell growth and whether such actions are dependent on receptor binding. Majority of breast cancer patients is prone to stress that governs the disease outcome. The effects of stress on the immunocompetence are mediated through central neuronal and peripheral sympathetic systems. The studies from our laboratory provide further evidence for the importance of neural-immune interactions.

Reportable outcomes

ThyagaRajan S, Madden KS, Felten SY, Felten DL. Inhibition of tumor growth by L-deprenyl involves neural-immune interactions in rats with spontaneously developing mammary tumors. Anticancer Res., (paper in press).

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Figure Legends

Figure 1. Effects of i. p. administration of 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl for 9 weeks on the average tumor diameter in rats with spontaneously developing mammary tumors. Sprague-Dawley female rats (12-mo) were housed in the animal quarters and palpated for the development of tumors every week. After the development of tumors, animals were treated with saline or deprenyl for 9 weeks. *Significantly ($P<0.05$) different from the Saline group.

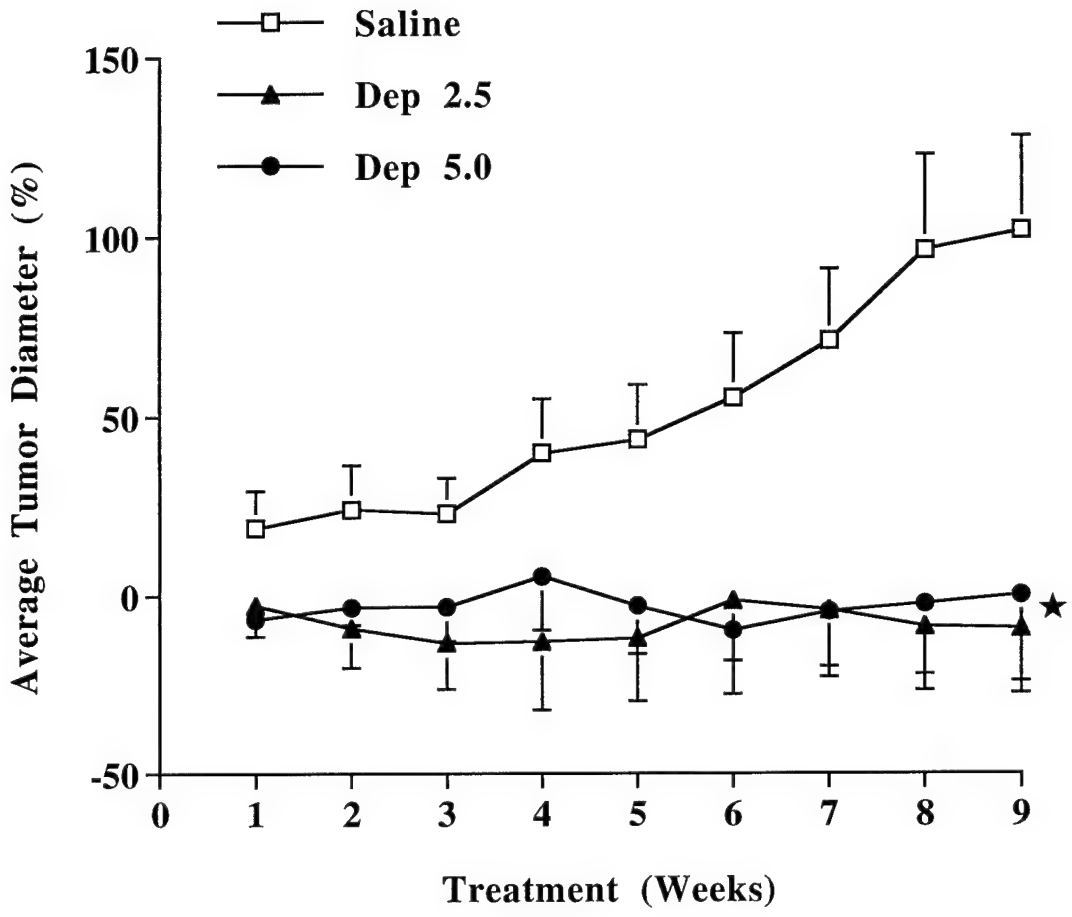
Figure 2. Effects of i. p. administration of 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl for 9 weeks on the average tumor number in rats with spontaneously developing mammary tumors.

Figure 3. Effects of i. p. administration of 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl for 9 weeks on the body weight of rats with spontaneously developing mammary tumors.

Figure 4. IFN-γ production by spleen cells from rats with spontaneously developing mammary tumors after 9 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 1.25 μg/ml of Con A for 24 hrs. Supernatants were tested for IFN-γ by ELISA. *Significantly ($P<0.05$) different from Saline group.

Figure 5. Con A-induced T lymphocyte proliferation by spleen cells from rats with spontaneously developing mammary tumors after 9 weeks of treatment with 0, 2.5 mg, or 5.0 mg/kg BW/day of deprenyl. Spleen cells were incubated with 0, 0.3, 1.25, or 5 μg/ml of Con A for 72 hrs. Proliferation of T lymphocytes at 5 μg/ml of Con A was enhanced in rats that were treated with 5.0 mg/kg deprenyl.

Figure 1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Average Tumor Diameter (%)
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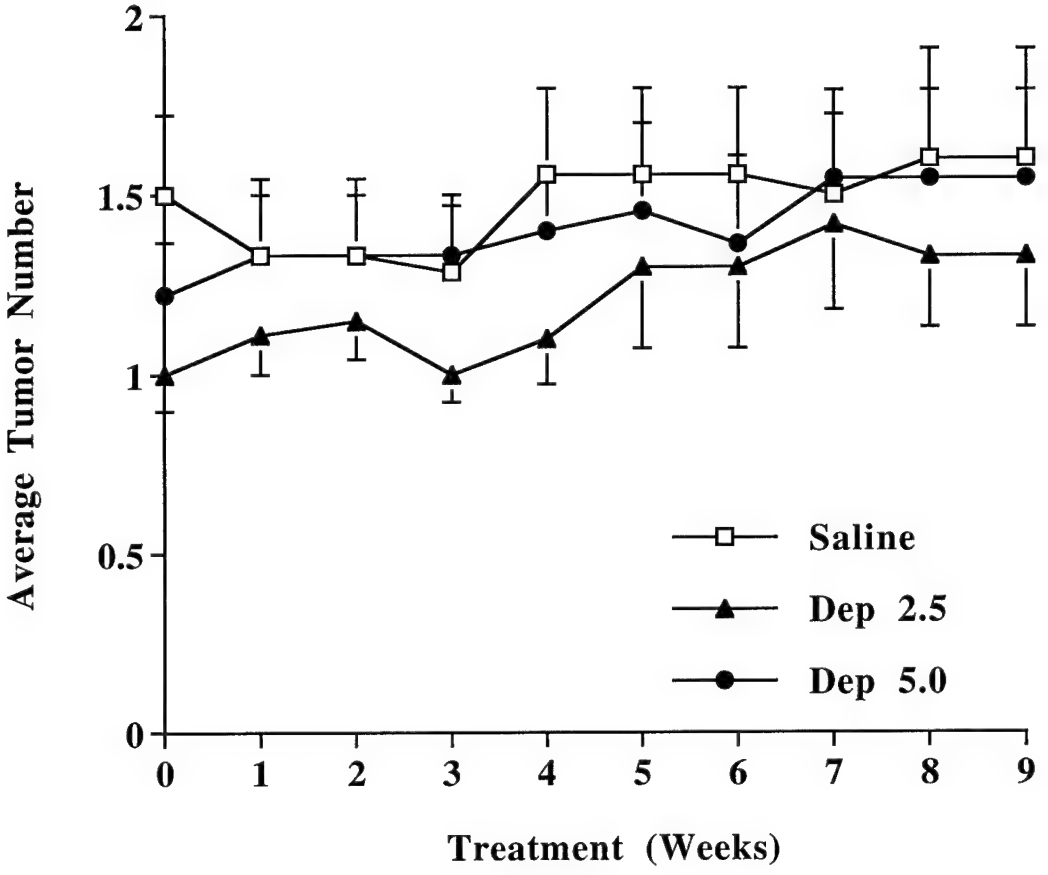
Figure 2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Treatment (Weeks)
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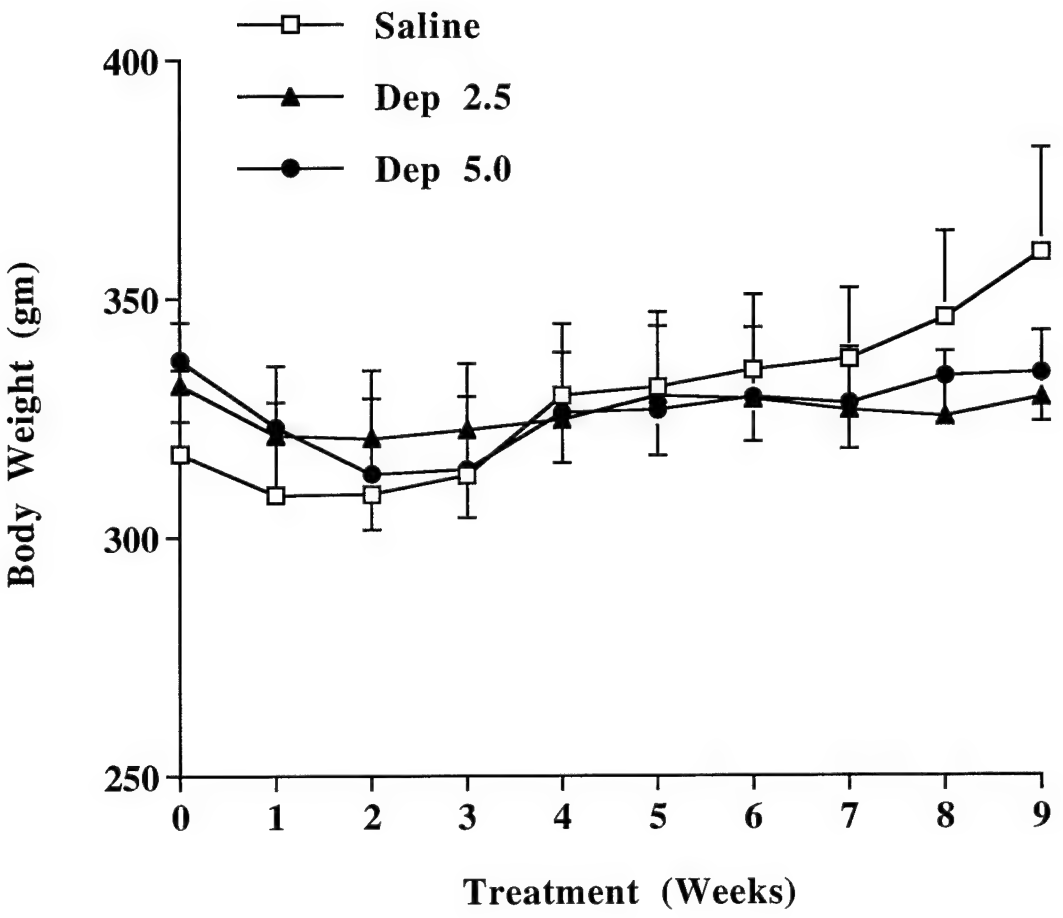
Figure 3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Treatment (Weeks)

Figure 4

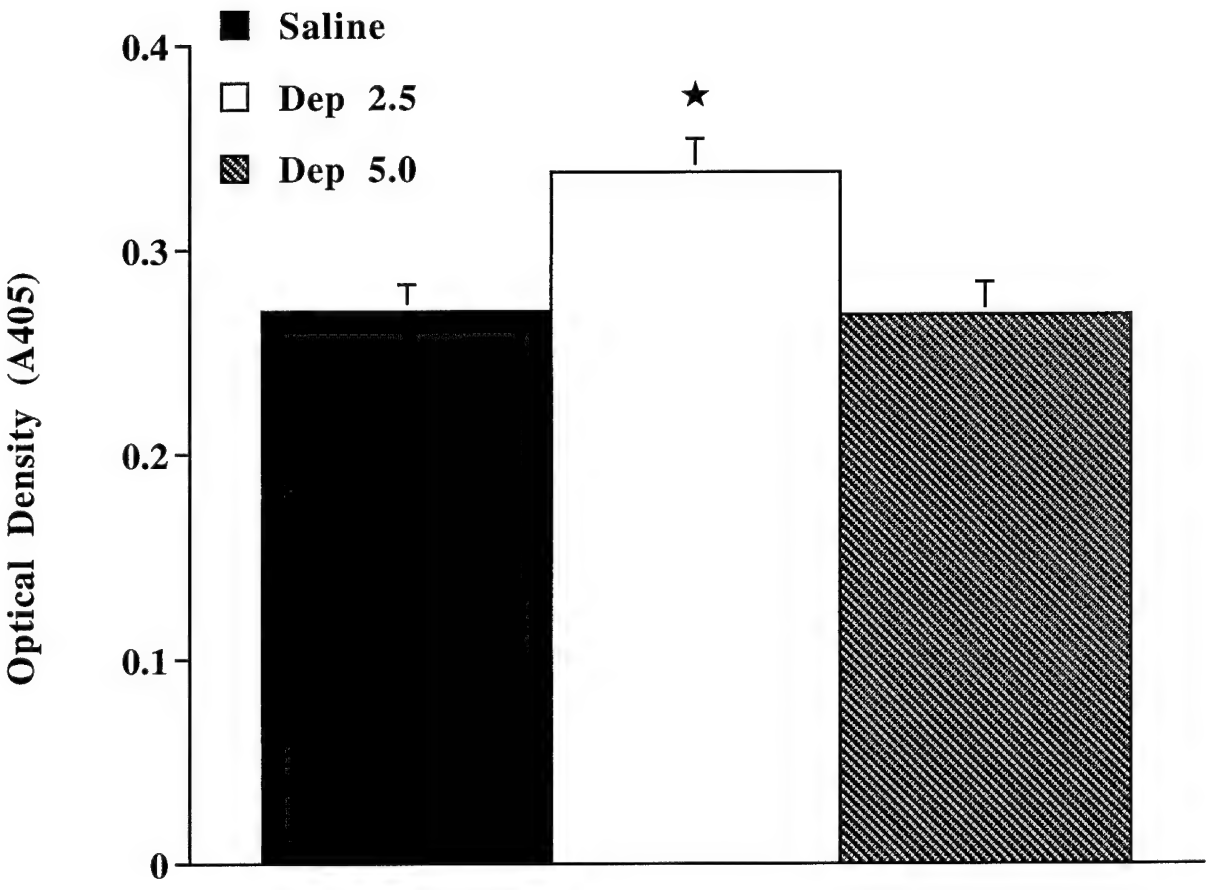

Figure 5

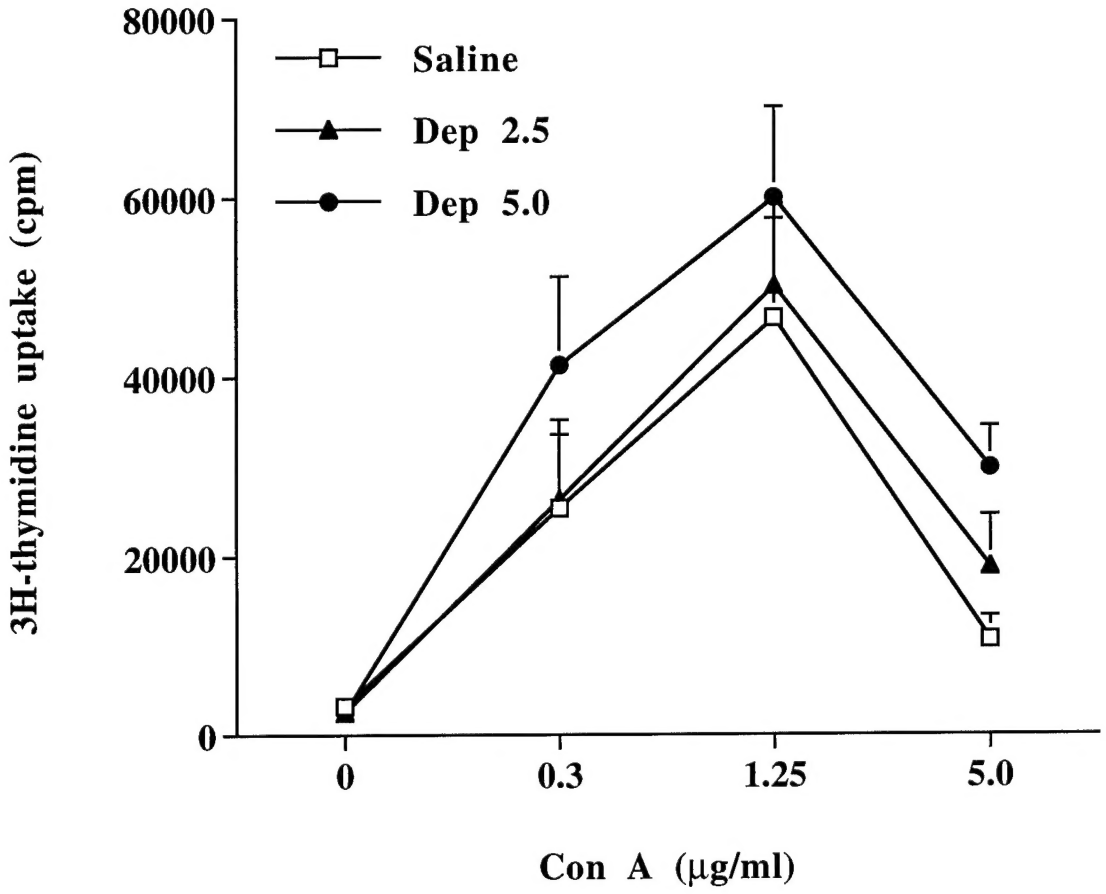
80000
60000
40000
20000
0
Saline
Dep 2.5
Dep 5.0
3H-thymidine uptake (cpm)
0
0.3
1.25
5.0
Con A (µg/ml)

Table 1. Spleen lymphocyte population from rats with spontaneously developing mammary tumors.

Groups	% sIgM+	% CD4+	% CD8+	% NK+
Saline	40.8±2.4*a*	39.3±2.4	12.9±0.5	5.3±0.8
Dep 2.5	42.3±1.5	39.9±2.4	17.1±1.5*b*	4.9±0.5
Dep 5.0	39.9±2.8	41.8±2.3	16.1±1.2*b*	4.5±0.6

*a*All values are Mean±SEM

b Significantly (P<0.05) different from Saline

Table 2. Effects of deprenyl treatment on splenic norepinephrine (NE) concentration in rats with spontaneously developing mammary tumors.

Groups	Whole Spleen wt. (g)	NE concentration in Spleen				NE content in Whole spleen (pmoles/mg wet wt.)
		Hilar region		End region		
		pmoles /mg protein	pmoles /mg wet wt.	pmoles /mg protein	pmoles /mg wet wt.	
Saline	0.75±0.11[a]	9.6±1.9	372.6±79.9	13.4±2.9	485.5±113.1	2245.9±460.7
Dep 2.5	0.72±0.06	15.1±1.6[b]	552.4±52.7[b]	14.0±2.7	520.7±69.1	2434.3±294.1
Dep 5.0	0.67±0.04	19.9±3.7[b]	683.3±115.9[b]	21.6±3.3	765.3±111.1	3320.8±490.8

[a] All values are Mean±SEM

[b] Significantly ($P<0.05$) different from Saline

Table 3. Effects of deprenyl treatment on catecholamines, indoleamine, and their metabolites in the medial basal hypothalamus (MBH) of rats with spontaneously developing mammary tumors.

Groups	NE	DOPAC	DA	5-HIAA	5-HT
Saline	181.2±13.1[a]	24.8±3.5	43.5±2.4	68.2±6.9	63.1±2.9
Dep 2.5	261.3±21.3[b]	14.7±1.4[b]	50.2±4.5	69.6±3.9	98.7±6.1[b]
Dep 5.0	306.9±20.9[b]	8.5±1.8[b]	67.7±9.2[b]	58.9±5.3	142.5±12.5[c]

[a] All values are Mean±SEM

[b] Significantly ($P < 0.05$) different from Saline

[c] Significantly ($P < 0.05$) different from Saline and Dep 2.5